REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2003	Final (1 Jul 2001 - 31 Dec 2002)

4. TITLE AND SUBTITLE
Inhibition of PKR Activity is Required for Survival of Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0585

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

PKR is an interferon-inducible protein kinase, which has recently been discovered to have pleiotropic effects on the growth and differentiation of normal and neoplastic cells. We reported a direct correlation between PKR expression and differentiation in a variety of human tumors and in normal squamous epithelial, and an inverse association between PKR expression and proliferation in head and neck cancer.

The mechanisms by which PKR produced such effects are being intensively studied. Phosphorylation of its major substrate, eIF-2α, leads to a selective inhibition of protein synthesis. PKR also phosphorylates IκB, freeing NFkB to tranlocate to the nucleus and induce transcription of a number of gene products. PKR-induced apoptosis has recently been reported to be co-dependent on both the eIF2α and NFκB pathways in HeLa cells.

Breast cancer was utilized as a model system to determine the role of PKR in loss of hormone and growth factor dependence in human cancers, as it appears inactive or hypoactive, as determined by eIF-2α phosphyorylation in this system. We hypothesized that hormone deprivation induces apoptosis in dependent cell lines through a PKR: NFκB mediated process, but this pathway is abrogated in hormone-independent cell lines.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, apoptosis, estrogen, cell proliferation	14
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

AD_____

Award Number: DAMD17-01-1-0585

TITLE: Inhibition of PKR Activity is Required for Survival of Breast Cancer

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REPORT DATE: January 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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FINAL REPORT: Award Number DAMD17-01-1-0585
G. Kenneth Haines III, M.D.

Introduction:

PKR is an interferon-inducible protein kinase, which has pleiotropic effects on the growth and differentiation of normal and neoplastic cells. We reported a direct correlation between PKR expression and differentiation in normal epithelia and in a variety of human tumors, as well as an inverse association between PKR expression and proliferation in head and neck squamous cell carcinoma.

The mechanisms by which PKR produces such effects are being intensively studied. Phosphorylation of its major substrate, eIF-2α, leads to an inhibition of cellular protein synthesis, which could directly block cell proliferation and more indirectly induce cellular differentiation. Other mechanisms certainly play a role in PKR's physiologic effects, including NFκB and other cellular transduction pathways.

Our preliminary work identified breast cancer as an intriguing model for understanding the mechanisms by which PKR influences the cellular outcome (death vs. survival) of tumor cells upon deprivation of growth stimulatory hormones/cytokines. The effectiveness of with anti-estrogens like Tamoxifin and growth factor-receptor blocking antibodies like Herceptin, may be mediated in part through PKR-dependent pathways. Tumors refractory to such agents must prevent PKR from blocking the unregulated cell proliferation that characterizes these tumors. We had found that PKR expression is decreased in breast carcinoma, relative to non-neoplastic breast ductal epithelium adjacent to the tumor. The PKR that is present in these tumors appears to be inactive or hypoactive, as determined by eIF-2α phosphorylation and the limited correlation with cellular differentiation.

In this study, we hypothesis that hormone deprivation induces apoptosis in dependent cell lines through a PKR: NFκB mediated process. This pathway is abrogated in estrogen-independent breast carcinoma through 1) decreased PKR production, 2) decreased activation, and/or 3) direct inhibition of PKR activity. To test this hypothesis, we attempted to modify PKR levels in hormone-dependent and independent breast carcinoma cell lines through gene manipulation (transfection) and chemical treatment with known PKR activators and inhibitors. Measurement of cell proliferation, apoptosis, and phosphorylation of PKR substrates IκB and eIF-2α would determine if PKR was a key factor in abrogation of hormone or growth factor dependence in breast cancer.

Key Research Accomplishments:

This project was directed at using surrogate genetics to up regulate and down regulate the expression of PKR in human breast cancers, and to determine whether or not PKR was the controlling gene for growth and cellular differentiation. We had obtained the reported clone for PKR from Glenn Barber, University of Washington, and had shuttled the gene into our proposed expression plasmid [PKR (wt)-BKCMV] prior to the funding of this

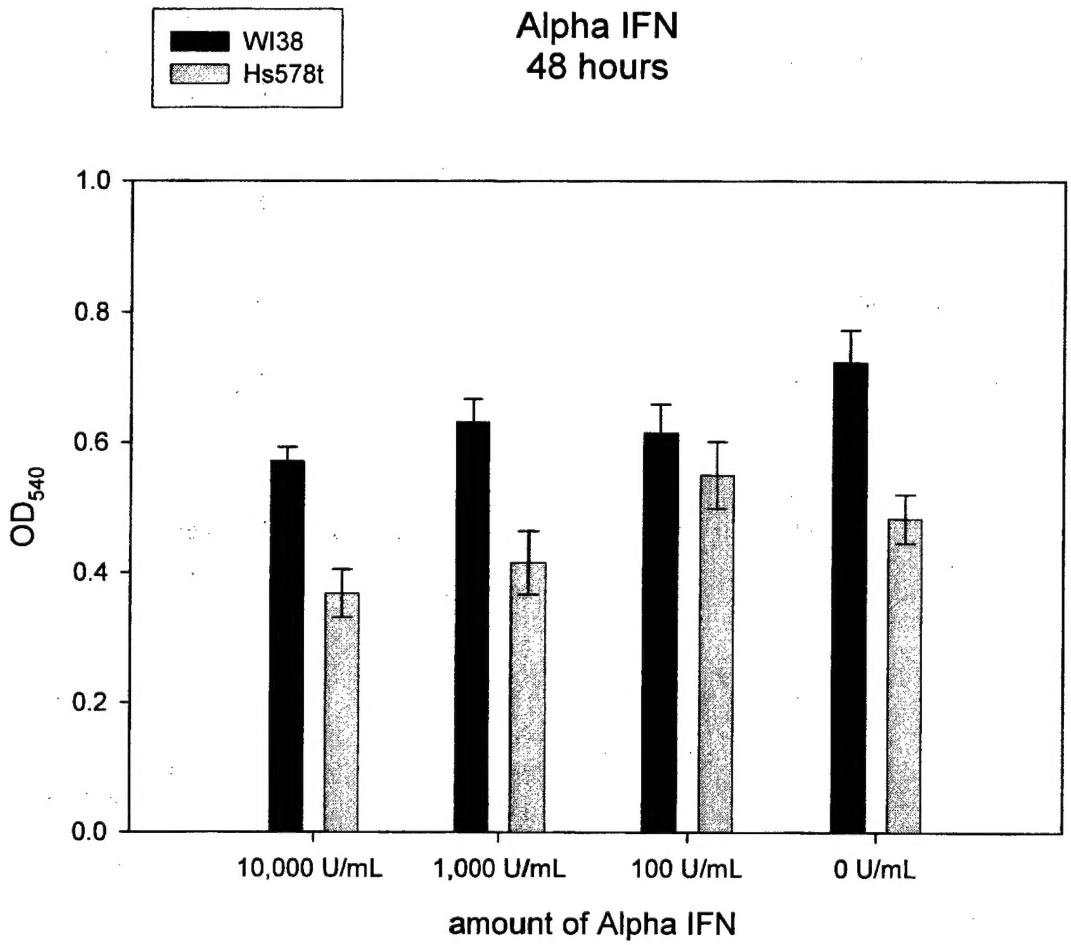
project. As part of this project we made the PKR- (k62)-BKCMV construct. Using both of these plasmids and the parent BKCMV plasmid, we set out to transfect, as proposed in the grant, the human breast cancer cell lines Hs578t (tumor) and Hs578 (normal). Our laboratory had routinely conducted tranfections using other cell lines and this as well as other parent plasmids and genes with success. After numerous attempts using Lipofectamine Reagent as the transfection reagent without success, we hypothesized that the transfection reagent was not optimal to this cell. We then set forth a series of experiments using two additional transfection reagents, Lipofectamine Plus Reagent and Effectene Transfection Reagent, as in our experience, the transfection reagent per each cell line was the most common and sensitive failure point in these types of experiments. When all three reagents failed to produce any clones, we began to suspect that there was a problem somewhere else in our system.

In order to rule out the possibility that the cell line as proposed was the problem, we used several cell lines including Detroit 562, KB-1, WI-38, and A549, with the above transfection reagents. We obtained no transfectants using all of these cell lines and the transfection reagent. This led us to believe that there was something fundamentally wrong with the plasmid constructs. We had previously cut and clone the insert given to us with comparable ease, and the insert/plasmid size/ratios were as expected.

Over the past five months (off grant) we have successfully used rtPCR to isolate our own PKR cDNA. We have successfully added (via PCR) restriction enzyme clone sites to the gene and plan to remake the proposed plasmids (Appendix I). Sequence data of the rtPCR product is consistent with the known sequence for PKR (Appendix II).

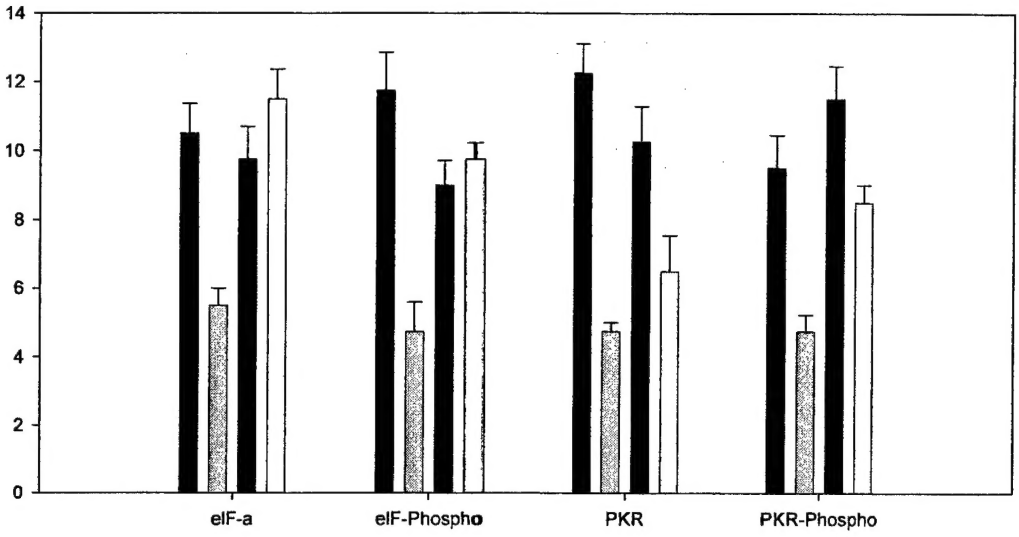
While working with our transfection system, we used chemical treatment to modify the quantity and activity of native PKR in a breast cancer cell line (Hs578t) and a non-neoplastic control (WI38 fibroblasts).

Incubation with alpha interferon for 48 hours had a modest inhibitory effect on growth of breast carcinoma cells. Interferon was only minimally inhibitory to WI38 fibroblasts.

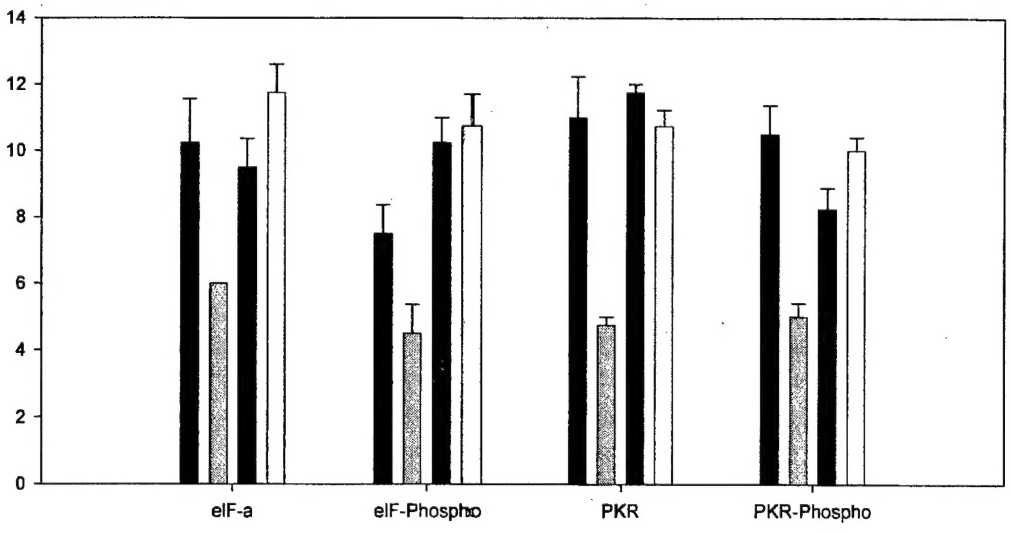


Alpha interferon treatment has a number of effects on cells. Additional experiments were needed to indicate document which effects may be attributed to PKR-mediated pathways. Slot blot 2 (below) shows the effect of interferon treatment on PKR and eIF2alpha protein levels in the breast cancer cell line Hs578t and WI38 fibroblasts. Aside from globally low protein levels in the 100 IU/ml treatment group (presumably a technical problem) we were not able to significantly alter the amount of PKR in this system.

SLOT BLOT (2)
Hs578t

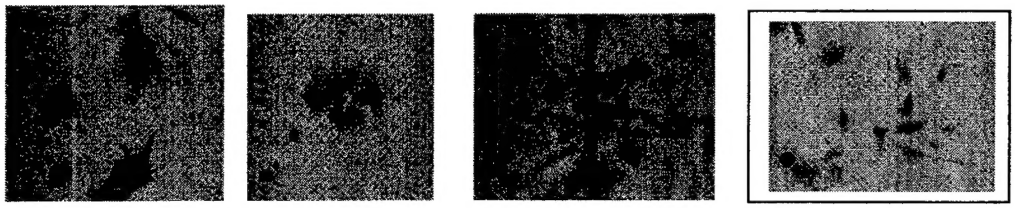


SLOT BLOT (2)
WI-38



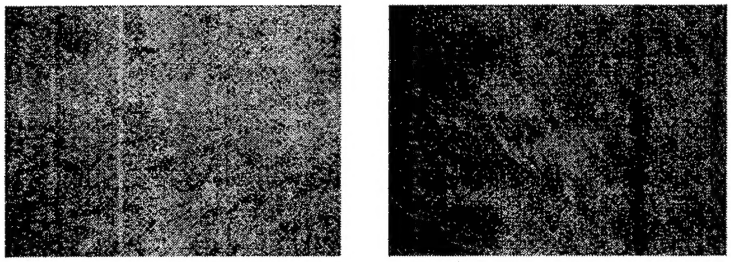
To collaborate these findings, we used an immunohistochemical approach in slide cultures. We did not detect a consistent change in PKR expression with alpha-interferon treatment in either the breast cancer cell line or fibroblasts.

PKR expression in Hs578t cells after 24° incubation with alpha IFN at doses:
0 IU/ml 100 IU/ml 1000 IU/ml 10000 IU/ml



Strong PKR expression (brown cytoplasmic staining) is seen with 0 and 1000 IU/ml IFN treatment. Lower levels of PKR are seen in the 100 and 10000 IU/ml cultures.

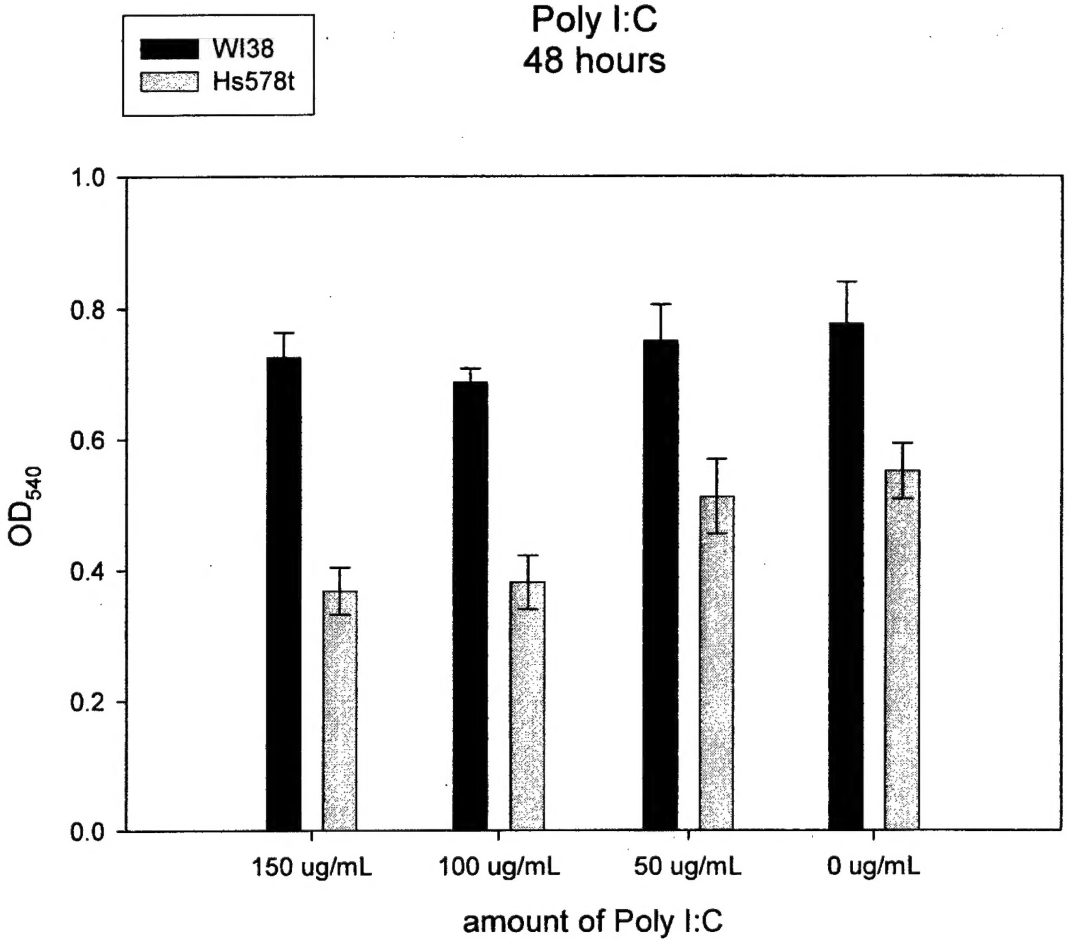
PKR expression in WI38 fibroblasts after 24° incubation with alpha IFN at doses:
0 IU/ml 100 IU/ml



PKR expression is minimal in non-neoplastic fibroblasts. A modest amount of PKR induction is seen in the 100 IU/ml group.

The lack of PKR induction at 24-48 hours in the breast carcinoma cells may have been related to an interferon-induced (PKR-mediated) decrease in protein synthesis. Short-term changes in PKR expression (30 minutes to 4 hours) would not be detected with this experimental design.

To determine the functional activity of PKR in breast cancer cells, we incubated Hs578t breast cancer and WI38 fibroblast cells with the PKR activator, poly(I:C), a synthetic dsRNA. We were able to show a modest inhibition of cell proliferation in the fibroblasts, but no significant difference in the carcinoma cells.



As would be expected with the insensitivity of the breast cancer cells to PKR activation, the addition of the PKR inhibitor, 2-aminopurine to cultures grown in 150 ug/ml Poly (I:C) had no effect on proliferation of the tumor cells.

Reportable Outcomes: None (yet).

Conclusions:

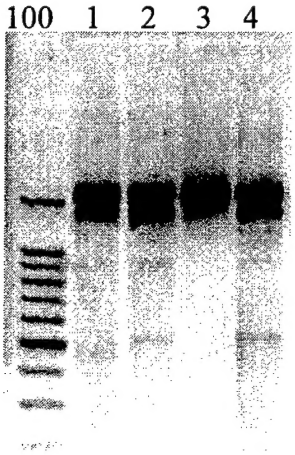
While we have to be successful in completing the proposed project, we are diligently working to complete these research goals. We will soon have an authentic and function PKR construct, which we intended to make available to all researchers. (Pending funding, we will use site directed mutagenesis to produce the k62 mutant.) Once we have these new constructs we will be able to rapidly complete the proposed objective using the above mentioned cells lines and transfection reagents.

Reference: None

Appendices:

Appendix I – Gels of rtPCR

Gel 1.



100: 100bp ladder

Lane 1. WI38 only RNA reverse transcribed using PKR Downstream primer. Amplified with PKR primers.

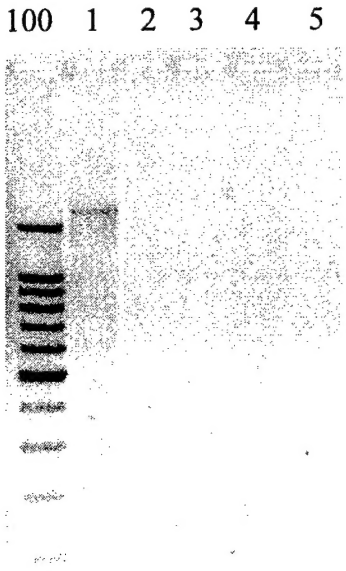
Lane 2. A549 RNA reverse transcribed using PKR Downstream primer. Amplified with PKR primers.

Lane 3. WI38 only RNA reverse transcribed using dT primers from the kit . Amplified with PKR primers.

Lane 4. A549 RNA reverse transcribed using dT primers from the kit . Amplified with PKR primers.

Lanes 5 and 6 (data not shown). Negative Control, WI38 only RNA and A549 RNA respectively, reverse transcribed using dT primers from the kit. Amplified with PKR primers. No reverse transcriptase was added to the reverse transcriptase reaction. No bands were seen.

Gel 2.



100: 100 bp marker

Lane 1. Lane 3 from Gel 1 diluted 1:100 amplified with PKR primers with an extension including a SalI restriction site.

Lane 2. PCR control = primer + template, but no taq was added to the rxn mix.

Lane 3. PCR control = no primers were added to the rxn mix.

Lane 4. PCR control = no template was added to the rxn mix.

Lane 5. PCR control = no dna was added to the rxn mix, including primers and template.

Primers

Primers used for RT PCR

GGAAGAAGAAATGGCTGGTG → PKR Upstream primer

GGGCTCTAACATGTGTGTCGT → PKR Downstream primer

Primers used to add SalI restriction site

GGCGTGG**GTCGAC**GGAAGAAGAAATGGCTGGTG → PKR SalI Upstream

GGCGTGG**GTCGAC**GGGCTCTAACATGTGTGTCGT → PKR SalI Downstream

Appendix II- Sequence data of rtPCR

Big Upstream Product(RID: 1050932318-010228-26093)Refers to the bigger of the two predominate bands on the gel = by sequence similarity this band is PKR

GGCTNNGANAGTTANNTGAAACTGTNNTACNTGACACGNACAGAAAGCAGGGGAGGTAGATACTTAAATNT
CAAGNAACTGCCTAATTCAGGACCTCCACATGATAGGAGGTTTACATTTCAAGTTATAATAGATGGAAGAG
AATTTCCAGAAGGTGAAGGTAGATCAAAGAAGGAAGCAAAAAATGCCGCAGCCAAATTAGCTGTTGAGATA
CTTAATAAGGAAAAGAAGGCNGTTAGTCCTTTATTATTGACAACAACGAATTCTTCAGAAGGATTATCCAT
GGGGAATTACATAGGCCTTATCAATAGAATTGCCCAGAAGAAAAGACTAACTGTAAATTATGAACAGTGTG
CATCGGGGGTGCATGGGCCAGAAGGATTTCATTATAAATGCAAAATGGGACAGAAAGAATATAGTATTGGT
NCAGGTTCTACTAAACAGGAAGCAAAACAATTGGCCGCTAAACTTGCATATCTTCAGATATTATCAGAAGA
AACCTCAGNGAAATCTGACTACCTGTCCTNTGGTTCTTTTGCTNCTACGTGTGANTCCCAAANCNACTCTN
TNGTGACCNNCN

~580bp

Big Downstream product (RID: 1050959946-019693-18542) Refers to the bigger of the two predominate bands on the gel = by sequence similarity this band is PKR

GGTCTTTCTTTTTCCCACAGANACAACCGCTGCCTTAGTATTTCAGATGTGTTAGGTCGATCCTCAGGTTT
CTTTGAGCAGTAATTTCTGTAGAAGAGTTTTTTCTTTTTTATCAAATATATCTGAGATGATGCCATCCCGT
AGGTCTGTGAAAAACTTTGATGTTTCAAAAGCAGTGTCACATACATGAAGAAGTTCAGCAAGAATTAGCCC
CAAAGCGTAGAGGTCCACTTCCTTTCCATAGTCTTGCGAAGAAATCTGTTCTGGGCTCATGTATCGCAAAG
TTCCCTTACTCCTTGTTCGCTTTCCATCATTTTTCAGAGATGTTACAAGTCCAAAGTCTCCAATCTTTACT
TGTTTTGTATCTACTAAGAATATATTACTTGGCTTAAGATCTCTATGAATTAATTTTTTTGAATGTATATA
ATCCACCCCTTTTGTTATTTGTTCAAAGAGTTCCAAAGCCAAAACTTTGTCTAGTTTCTCGCCTCTTCTTT
TTTCAATCCATTGNTCCAAGGTCCCTTTATCACAGAATTCCATTTGGATGAAAAGGCACTTAGTCTTTGAC
CTTGAACTATTTTTGCTGTTCTCAGGATCATAATCACTGCTCTCAAGAGAATCATCAC

Small Downstream Product (Refers to the smaller of the two predominate bands on the gel)

CAAGNGGTNGTGCTTTTTGNCTGCGAACAACCGCAGCACTGGCTCTTCATACAGNCAGTAGGGTCATTTTC
ANCAATACCCAGGACCAAGTTTGATCATCCTGTANGATCCTGTTAGCATGTGTCTGGGGATCTTCCAGACT
GAAGCCAGAAGACAGGAGCGCAGTTTCATAAAGCAAGATGACCAGATCCTTCACAGACTTGTCGTTCTTAT
CAGCCTCTGCCTTTTGCCTTAAGGTCTCAATAATGGAATGGTCAGGGTTTATCTCCAGGTGTTTCTTTGCT
GCCATGTAACCCATTGTTGAGTTGTCTCTTAGGGCTTGAGCTTTCATGATTCTCTCCATGTTTGCTGTCCA
GCCATATGTGCTTGTGACAATACAGCATGGAGATGTCACCAATCGGTTNNGACACAACCACCTTTTCAACT
TTTTTCTCCAATATGTCTTTCATGATTTTGCAGAGGTTCTCAAACTTTGTTTTTTTCTCTTCCTGCTTCTT
TTTCTCTTCTTCATCCTCTGGAAGNTCCAGGCCTTCTTTGGTGACTGACACTAAAGTCTTCCCCTCAAATT
CCTTCAGCTGTTGGACACAGTACTCATCAATGGGCTCAATCATATAGATCACTTCTAAGCCATGTTTCCGA
ANACGTTCCACAAAGGCTGAGTTAGCNACCTGG

Small Upstream Product (RID: 1050933032-016599-17205) (Refers to the smaller of the two predominate bands on the gel)

AGTNCNNNGGGTGGGTANGTNNGTAANGGGCTAGCNCGGTAAGAAAGAAAANGGCTTGGANGGCAGAACCC
ATTTTGNGACCGGCAAANCCNGACCANTNCNACTAATGAGGAGGACGGAGANTTCTATAGGAGCTTGACCA
ANGACNGGGAAGATCACTNGGCAGGGAAGCNTNNTTCAGATGAAGGACAGNTGGAATTCANAGCCCTTCTA
TTTGTCCCACGACGTGCTCCTTTTGNTCTGTNTGAAAACAGNNNGANAANCANCAATNTCAAATNGTGTGT
ACGCAGAGTNTTCATCATGGATAACTGTGAGGAGCTAATCCCTGAATACCNTGANCNNCATTAGAGGGGAG
GTAGACTCNGANGATCTACCTCTAAACATATGCCGAGAGANGNNACAACAAAGCACANTTTTGAAAGNTAN
CAGGAGAAATCGCCCANAAAATGCTNANAACNAANCNCNGGGC